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Trichloroethylene Exposure in Mid-Pregnancy Decreased Fetal Weight and Increased Placental Markers of Oxidative Stress in Rats

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Abstract

Although epidemiology studies have associated maternal trichloroethylene (TCE) exposure with decreased birth weight and preterm birth, mechanistic explanations for these associations are currently lacking. We hypothesized that TCE targets the placenta with adverse consequences for pregnancy outcomes. Pregnant Wistar rats were exposed orally to vehicle or 480 mg TCE/kg body weight from gestational days (gd) 6-16, and tissues were collected on gd16. Exposure to TCE significantly decreased average fetal weight without reducing maternal weight. In placenta, TCE significantly increased 8-hydroxy-deoxyguanosine, global 5-hydroxymethylcytosine, and mRNA expression of *Tet3*, which codes for an enzyme involved in 5-hydroxymethylcytosine formation. Furthermore, glutathione S-transferase activity and immunohistochemical staining were increased in placentas of TCE-exposed rats. The present study provides the first evidence that TCE increases markers of oxidative stress in placenta in a fetal growth restriction rat model, providing new insight into the placenta as a potentially relevant target for TCE-induced adverse pregnancy outcomes.

Keywords

Trichloroethylene; fetal growth restriction; oxidative stress; Wistar rats; glutathione S-transferase; 8-hydroxy-deoxyguanosine; 5-hydroxymethylcytosine

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Conflict of interest

The authors declare that they have no conflict of interest.

1. Introduction

Trichloroethylene (TCE) is a chlorinated organic solvent that has been used in commercial applications such as metal degreasing, paint stripping, and dry cleaning. In the United States, 2.5 million pounds per year of TCE were used in 2011, while the global usage was 945 million pounds per year [1]. TCE is a widespread environmental pollutant due to improper disposal [2], and is among the most frequently detected United States Environmental Protection Agency (US EPA) regulated drinking water contaminants, detected in 4.5% (groundwater source) to 15% (surface water source) of US public water supplies [3]. In addition, TCE exposure can occur in occupational settings, with an estimated 3.5 million workers in the United States exposed to TCE with short-term exposure levels in air ranging from 1.3 mg/m^3 to 1,084 mg/m^3 (the highest mean concentration was reported for a degreasing operation) [4].

Metabolism of TCE plays an important role in its toxicity, with liver and kidneys being key sites of TCE bioactivation and target organs of TCE toxicity [5-7]. TCE is metabolized via two main pathways: cytochrome P450-mediated oxidation with trichloroacetic acid and dichloroacetic acid as major bioactive metabolites, and glutathione conjugation with subsequent metabolism to form *S*-(1,2-dichlorovinyl)-L-cysteine (DCVC) as a major bioactive metabolite (Lash 2000). The placenta expresses key TCE metabolizing enzymes including CYP2E1 and glutathione-*S*-transferase (GST) (Lash 2000). TCE is classified as a known human carcinogen by the International Agency for Research on Cancer (IARC) and the National Toxicology Program (NTP), based on strength of evidence of TCE as a kidney carcinogen [8-10]. TCE exposure increases cellular generation of reactive oxygen species (ROS), with evidence linking TCE-induced oxidative stress with toxicity in rat kidney [11], rat and mouse liver [12, 13], mouse brain [14, 15], and mouse immune cells [14, 16, 17]. Moreover, the nephrotoxic TCE metabolite *S*-(1,2-dichlorovinyl)-L-cysteine (DCVC) increases indicators of oxidative stress in kidney [18-21] and placental cells [22, 23].

Recent epidemiology reports indicate significant associations between exposure to TCE and adverse pregnancy outcomes that include decreased birth weight and preterm birth [24, 25]. TCE has been shown to cross the placenta in humans [26, 27]. Although the placenta plays key roles in fetal growth, development, and pregnancy [28], investigation of TCE-induced toxicity in placental cells has been limited to the human placental HTR-8/SVneo cell line in vitro [22, 23, 29]. Furthermore, the potential for in vivo TCE exposure to induce oxidative stress in the placenta has not been previosuly reported, to our knowledge, although the TCE metabolite DCVC stimulates generation of ROS in placental HTR-8/SVneo cells [22, 29].

Because the placenta is the essential interface between mother and fetus, changes in its function can significantly impact pregnancy outcomes [30]. The goal of the current study was to identify effects of TCE exposure during pregnancy on pregnancy outcomes and biomarkers of oxidative stress in the placenta of Wistar rats.

2. Materials and methods

2.1. Materials

Trichloroethylene (99% pure) was purchased from Sigma Chemical Company (St. Louis, MO, USA). RNAlater, RNeasy Plus Mini Kit and QIAamp DNA Mini Kit were purchased from SABiosciences (Valencia, CA, USA). The Colorimetric 8-OHdG DNA Damage Quantification Direct kit and MethylFlash Hydroxymethylated DNA Quantification kit were purchased from Epigentek (Farmingdale, NY, USA).

2.2. Animals

Animal experiments were approved by the University of Michigan Institutional Animal Care and Use Committee (Protocol #PRO00006721) and performed in accordance with all state and federal regulations for use of vertebrate animals. Timed-pregnant Wistar rats between 60-90 days of age weighing 200-250 grams were obtained from Charles River (Portage, MI, USA). The day after copulation was designated as day 0 of pregnancy. Rats were shipped at gestational day (gd) 2 and individually housed in a controlled environment with a 12-hour light/dark cycle. Dams were fed standard rat chow (Purina 5001) and water *ad libitum*.

2.3. Exposure and Experimental Design

Rats were administered TCE daily from gd 6-16 using vanilla miniwafers [31]. We chose this gestational age range to include TCE exposure of placenta from its earliest stages, and we terminated the experiment on gd 16 to allow us to examine placenta without potential confounding from rats going into early labor. Oral exposure was chosen as a route relevant for human environmental TCE exposure, and wafers were chosen instead of gavage to minimize stress. Rats were fed wafer only (vehicle controls) or wafer with 480 mg TCE/kg body weight between 8:00-9:00 am daily. The TCE dose was selected because 400-500 mg TCE/kg/d was previously shown to stimulate oxidative stress in rat liver [12]. Dams were weighed daily and the amount of TCE added to the wafer was adjusted based on the individual daily body weight of the dam. To ensure that the wafers were eaten quickly, rats were trained to eat the wafer without TCE over three consecutive days preceding TCE exposure. Prior to administration, rats were housed for 1 h without food, then TCE (undiluted) was pipetted onto a miniwafer that was immediately offered to the rats and readily consumed. Exposure on gd 16 occurred approximately 2 hours prior to euthanasia. Exposures were conducted in two blocks. The first block included eight TCE-exposed rats and seven controls (it was discovered that one rat in the control group was not pregnant). The second block included three rats in the control group and two rats in the TCE treatment group for a total N=10 in each treatment group.

2.4. Dissections

Data were collected from all dams for measures of litter size and fetal body, maternal body, liver, and kidney weights. Rats were euthanized at gd 16 with carbon dioxide followed by cardiac exsanguination.

Dams were euthanized alternating between controls and TCE-treated rats, conducted within a 4-h period in the morning. The uterine horn was removed and examined for resorbed or

dead fetuses. Fetal weights and litter size were recorded as well as maternal body, kidney, and liver weights. Placentas were snap frozen in liquid nitrogen and then stored in the −80°C freezer or in RNAlater reagent for future analysis.

2.5. Assessment of DNA base modifications

Genomic DNA was extracted from placental tissue of dams in the first exposure block (7 control dams and 8 TCE-exposed dams; 3 placentas per litter). Each tissue sample was weighed and then homogenized using a FastPrep-24 tissue and cell lyser (MP Biomedicals, Solon, OH, USA). DNA was extracted from the homogenized tissue using a QIAmp DNA Mini kit (Qiagen, Germantown, MD, USA) according to the manufacturer's protocol. Concentration and purity of the extracted DNA was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE, USA). The samples were stored at −80°C until assayed for levels of 8-hydroxy-deoxyguanosine (8-OHdG), 5-hydroxymethylcytosine (5-hmC), and 5-methylcytosine (5-mC).

Levels of 8-OHdG, 5-mC and 5-hmC were assessed in placental samples using the EpiQuik 8-OHdG DNA Damage Quantification Direct Colorimetric kit, MethylFlash Methylated DNA Quantification kit (Colorimetric) and MethylFlash Hydroxymethylated DNA Quantification kit (Epigentek, Farmingdale, NY), respectively, following the manufacture's protocols. For the 5-mC assay, placental DNA was pooled on a litter basis prior to assay. For the 8-OHdG and 5-hmC assay, DNA of each placenta was analyzed and then the data were averaged per litter. The same 3 placentas used in the 5-mC assay were also used in 5-hmC assay. Briefly, genomic DNA was added to strip wells that were pretreated to have a high affinity for DNA. Then, capture and detection antibodies were used to determine 8-OHdG or the methylated and hydroxymethylated fractions of the DNA. The absorbance was read at 450 nm on a Molecular Devices SpectraMax Gemini M2e spectrophotometer. The experiment was carried out in triplicate. Relative quantities of 5-mC and 5-hmC were calculated based on the manufacturer's guidelines. In addition, the same DNA samples used with the MethylFlash Hydroxymethylated DNA Quantification kit were also used to conduct a LUminometric global methylation assay (LUMA) according to Sant et al. [32] (data not shown).

2.6. Immunohistochemical detection of GST in rat placenta

Placentas were fixed in 10% formalin (Fisher) for a minimum of 48 h. Immunohistochemistry was performed by the University to Michigan Histology Core. Briefly, unstained 5-μm sections were cut from paraffin-embedded, formalin-fixed tissue using a rotary microtome and mounted on glass slides. Placenta GST detection was performed using a commercially available primary rabbit polyclonal antibody against GST-pi (GWB-BBP465, GenWay Biotech). We analyzed for GST-pi because it is the GST isoform that is predominantly expressed in the placenta; the other GST isoforms are either not expressed in the placenta (GST-alpha) or are expressed at low concentrations (GST-theta and GST-mu) [33]. To analyze for protein expression of GST-pi in the placenta, heat-induced antigen retrieval was performed with citrate buffer (pH 6) for 10 min. Immunoperoxidase staining was completed on a Dako AutoStainer at room temperature using a LSAB2 visualization kit (Agilent Dako). Briefly, peroxidase block was followed by a 30-min

incubation with primary antibody at a dilution of 1:1500 rabbit polyclonal. Samples were then incubated sequentially with a biotinylated link antibody (produced in goat) at a dilution of 1:500 for 30 min, streptavidin-HRP conjugate incubation for 20 min, and 3,3′-diaminobenzidine chromogen solution for 10 min. As an antibody control, placental sections were incubated with secondary antibody only. Microscopy imaging was conducted using Nikon Elements. One placenta per litter was harvested from dams in the first exposure block (2 control and 3 TCE-exposed rats) and second exposure block (3 control and 2 TCE-exposed rats). For each placenta, 5-6 images were analyzed, with one image selected in each of the four quadrants and one or two in the middle of the image field.

2.7. Glutathione S-transferase (GST) and γ-glutamyltransferase (GGT) enzyme activity assays

Activity levels of GST and GGT were measured in a subset of rat placentas collected from dams in the first exposure block (1 control and 2 TCE-exposed rats) and second exposure block (3 control and 2 TCE-exposed rats), 3 placentas per litter. Additional litters were not included due to limitation of tissue availability. The placentas were rinsed in phosphate-buffered saline (PBS), pooled, and homogenized in 2 mL cold PBS with 2 mM EDTA. The homogenized tissues were then centrifuged at 10,000 × g for 15 min at 4°C. The tissue extracts were collected and stored at −80°C and assayed the next day.

GST enzyme activity was assayed according to Habig et al. [34], as modified by the Glutathione S-Transferase Assay kit (Cayman Chemical; Ann Arbor, MI, USA) for 96-well plates. GST activity was determined by adding 10 µl of 1-chloro-2,4-dinitrobenzene, 20 µL GSH, and 20 µL placental tissue extract to 150 µL GST buffer (containing 100 mM potassium phosphate and 0.1% Triton X-100). A SpectraMax M2e Multi-Mode Microplate Reader (Molecular Devices) was used to quantify formation of 2,4-dinitrophenylglutathione kinetically at 340 nm ($\varepsilon = 9.6\ mM^{-1}\ cm^{-1}$). GST activity was calculated according to the manufacturer's guidelines. One unit of enzyme activity is defined as the amount of enzyme that forms 1.0 nmol of S-2,4-dinitrophenyl GSH/min at 25 °C. The level of protein was determined by the bicinchoninic acid (BCA) protein assay (Thermo Scientific, Waltham, MA, USA), using bovine serum albumin as a standard.

GGT enzyme activity was measured according to the method by Orlowski and Meister [35] as modified for the GGT Activity Colorimetric Assay kit (GenWay Biotech, San Diego, CA, USA) for 96-well plates. Briefly, 90 µL of γ-glutamyl-p-nitroanilide substrate was added to 10 µL of supernatant from placental tissue homogenates in a 96-well plate. The plate was then incubated at 37°C and absorbance was measured kinetically using a spectrofluorometer at 410 nm for appearance of p-nitroanilide (molar extinction =8800 $M^{-1}cm^{-1}$). GGT activity was calculated according to the manufacturer's guidelines. One unit of GGT was defined as the amount of enzyme that generated 1.0 µmole of p-nitroanilide per minute at 37°C. Protein amount was quantified using the BCA assay (Thermo Scientific), with bovine serum albumin as a standard.

2.8. RNA extraction and sample preparation

RNA was extracted from the same placentas used for DNA base modification assays. Placentas were dissected from the uterus, weighed, and a 25-mg sample was homogenized using a FastPrep-24 tissue and cell lyser (MP Biomedicals, Solon, OH, USA). The RNA was extracted using an RNeasy kit (SABiosicence) according to the manufacturer's protocol. RNA concentration and purity were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE, USA).

2.9. *Analysis of placental mRNA expression of* Tet2 *and* Tet3 *genes*

Placental expression of *Tet2* and *Tet3* genes was assessed using real time quantitative reverse transcription PCR (qRT-PCR). Aliquots of 1 μg of mRNA were used for cDNA synthesis using the RT2 First Strand kit (SABiosciences) following the manufacturer's protocol. qRT-PCR was performed using 12.5 μL of RT^2SYBR Green qPCR Master Mix, 1 μL of gene-specific primer target for *Tet2* and *Tet3* (SABiosciences, CA), 4 μL of cDNA template and 7.5 μL of nuclease-free H_2O, for a total volume of 25 μL. Samples were analysed using a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The qRT-PCR was run with an initial denaturation step of 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, and 5 s at 60°C. The ΔΔCt method [36] was used to quantify gene expression. To minimize error due to normalization [37], the target gene was normalized to the geometric mean of three non-differentially expressed housekeeping genes β2-microglobulin 5′ CGTGCTTGCCATTCAGAAAACT and 5′ GGTGGGTGGAACTGAGACAC 3′, β-actin 5′AAGCCGGCCTTGCACAT 3′ and 5′ CGCCACCAGTTCGCCA 3′, and T binding protein (Tbp) 5′GAATAAGAGAGCCACGAACAACTG 3′ and 5 ′ATTGTTCTTCACTCTTGGCTCCT 3′, using CFX manager software (Bio-Rad Laboratories), consistent with recommendations of the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines [38]. *Tet2 and Tet3* mRNA analyses were conducted on placenta collected from all dams in the first exposure block (7 controls and 8 TCE-treated; 3 pooled placentas per litter). All samples were analyzed in triplicate by qRT-PCR (technical replicates).

2.10. Statistical analysis

For all analyses, the dam was used as the statistical unit. Analysis for differences in average fetal weight used mixed model ANOVA in SPSS software (SPSS Inc., Chicago, IL, USA), with treatment as the fixed effect and litter as the random effect. Other data were analysed by t-tests using Graphpad Prism 5.0 (LaJolla, CA, USA). All data were expressed as mean ± SEM.

3. Results

3.1. Reproductive effects of TCE

All exposed rats were pregnant on the day of euthanasia except for one rat in the control group that was discovered earlier to not be pregnant. TCE exposure during pregnancy decreased average fetal body weight by 10% (Fig. 1A; $p<0.05$) with no significant change in

litter size (Fig 1B). The average maternal body weight of TCE-treated rats was not significantly different from controls (Fig 1C). In addition, maternal liver and kidney weights did not change with TCE exposure compared with controls (Supplemental Data, Fig. S1). No clinical signs of toxicity were observed over the course of the experiment.

3.2. TCE effects on placental DNA base oxidation and methylation

Levels of 8-OHdG in the placentas of TCE-treated rats were significantly increased by 42.2% compared with controls (Fig. 2; p=0.02). Likewise, TCE exposure increased global DNA levels of 5-hmC in placentas of TCE-exposed rats by 53.9% compared with controls (Fig. 2; p=0.005). However, global placental 5-mC levels were not significantly modified by TCE exposure (Fig. 2; p=0.84, not significant) using the commercial immunoassay kit, a finding corroborated by the LUminometric methylation assay (data not shown).

3.3. TCE effects on placental Tet gene

Because 5-hmC can be formed by Tet enzyme conversion of 5-mC to 5-hmC as well as by oxidation of DNA bases, we also assessed mRNA expression of the *Tet2* and *Tet3* genes. A statistically significant increase in mRNA expression for *Tet3* (p=0.02), but not *Tet2,* was observed (Fig. 3).

3.4. Assessment of placental GST and GGT

Exposure to TCE increased GST activity in rat placenta tissue 1.8-fold compared with controls ($p<0.05$, Fig 4A). Moreover, immunohistochemical staining indicated increased protein expression of GST-pi in TCE-treated rat placenta. In representative images showing the left basal and labyrinth zones on the fetal side of the placenta (Fig. 4), GST-pi antibody stained throughout the tissues in both the labyrinth and the basal zones of the placenta. However, the staining was visibly increased and darker within the labyrinth as well as the basal zones in placenta of TCE-treated rats (Fig. 4C) compared with control rats (Fig. 4B). In contrast to GST, activity for GGT was not significantly increased (Supplemental Data, Fig. S2).

4. Discussion

Despite reductions of release into the environment over recent decades, TCE exposure remains significant due to exposures in the workplace and contamination of water, soil, and air [2]. In 2014, TCE was reclassified by the International Agency for Research on Cancer (IARC) and the National Toxicology Program (NTP) as a known human carcinogen [8-10]. Although less studied than cancer risk, TCE is also linked to adverse pregnancy outcomes in humans [39].

Our finding that TCE exposure during pregnancy decreased fetal weight in Wistar rats is consistent with previous epidemiology studies that associated TCE exposure with decreased birth weight. Specifically, a study of pregnant women exposed to TCE-contaminated drinking water at Camp LeJeune found increased odds for small for gestational age (SGA) and decreased birth weight associated with TCE exposure [25, 40]. Similarly, Forand et al. found that maternal exposure to TCE from home vapor intrusion increased risk for fetal

growth restriction, low birth weight, and preterm birth in a New York State cohort [24]. Our results showing fetal growth restriction in rats exposed during pregnancy to TCE without overt toxicity to the dam, as indicated by lack of significant changes in maternal body and organ (liver and kidney) weights, support use of this animal model for studying placental TCE responses of potential relevance to fetal growth restriction. The reduction in average fetal weight we observed approximates the clinical definition of fetal growth restriction for human infants born below the 10^{th} percentile for gestational age [41].

Our finding of decreased fetal weight following oral exposure of pregnant Wistar rats to 480 mg TCE/kgd on gd 6-16 differs from a prior study with Sprague-Dawley rats that found no significant reduction in fetal weight on gd 21 following exposure to 500 mg TCE/kg/d delivered by gavage from gd 6-15 [42]. It is possible that differences between the strain of rat, gestational age of fetal assessment, and form of oral exposure (TCE on a wafer treat in our study versus gavage of TCE diluted in soybean oil) contributed to the different results for the effect of TCE on fetal weight. Although a prior study by Healy and colleagues reported that inhalation exposure of Wistar rats to 100 ppm TCE from gd 8-21 for 4 hours per day significantly reduced fetal weight at term by approximately 9% [43], differences in route of exposure, dose, and gestational age of fetal assessment limit further comparisons with our results.

In the rat placenta, TCE increased levels of 8-OHdG, consistent with prior studies in animals and humans. For example, levels of 8-OHdG were significantly higher in urine of TCE-exposed workers compared with controls [44, 45]. In a subchronic mouse study, exposure to 800 or 1000 mg TCE/kg/d by gavage increased liver thiobarbituric acid-reactive substances during the first 2 weeks only, and at the highest concentration of 1000 mg TCE/kg/d increased liver 8-OHdG concentrations at multiple time points throughout the 8-week study [13]. Toraason and coworkers showed that TCE exposure elevated levels of 8-OHdG and thiobarbituric acid-reactive substances in rat liver 24 h after a single i.p. injection of 500 mg/kg (lowest effective dose) [12]. Similarly, a single oral TCE dose of 2000 mg/kg increased thiobarbituric acid-reactive substances in rat liver, peaking 6 h after dose administration [46, 47]. Despite these reports that exposure to TCE increases 8-OHdG and other markers of oxidative stress, to our knowledge, no prior studies have explored TCE-induced markers of oxidative stress in placenta in vivo.

Glutathione S-transferases (GSTs) are a family of enzymes that catalyze conjugation of a variety of substrates with GSH, and increased GST expression can be part of the cellular antioxidant response to ROS [48]. As such, our observations that TCE exposure increased GST activity and GST-pi protein in the rat placenta are consistent with increased placental ROS, as well as with a prior report that TCE increased GST activity in the liver and lung of rats [49]. Our detection of the GST-pi protein in rat placenta is consistent with previous studies that found GST-pi in human placenta, as well as in kidney and the digestive tract [50]. Furthermore, our finding of increased expression of GST-pi protein in placenta of TCE-treated rats is consistent with a report that occupational exposure to TCE was associated with increased levels of GST-pi in the urine [51], although Bruning and colleagues found a positive association between exposure to TCE and urinary concentrations of GST-alpha but not GST-pi in a retrospective study [52]. In rat kidney cells, GST-alpha is

the primary isoform responsible for GST conjugation of TCE [53]: however, GST-alpha is not abundantly expressed in the placenta [33].

In addition to having a role in antioxidant response, GST is important for initiating glutathione-dependent metabolism of TCE [7]. Studies have linked the TCE glutathione pathway metabolite DCVC to oxidative stress as a mechanism of toxicity in kidney cells [18-21] and a human placental cell line [22]. Although there is a paucity of research regarding TCE metabolism by the glutathione conjugation pathway in placenta, studies have reported the ability of human placental GST to metabolize other toxicants [54, 55]. After conjugation by GST, subsequent biotransformation by GGT is necessary to produce DCVC. In contrast to prior reports that TCE increases GGT activity in kidneys of mice, rats, and humans [56], we failed to detect statistically significantly increased GGT activity in placenta of TCE-exposed rats. One possibility is that variability and small sample size (N=4) contributed to the lack of statistical significance for GGT in our experiment. Regardless, we detected placental GGT activity and its presence, along with TCE-stimulated placental GST activity, implies that the placenta has the capability to bioactivate TCE via the glutathione pathway. Given that DCVC induces oxidative stress in human placental cells [22], we suggest that this TCE metabolite may play a key role in stimulating oxidative stress in placenta. However, further studies are warranted to validate a link between TCE metabolism by the glutathione activation pathway and placental toxicity.

Our findings of increased 5-hmC and *Tet3* gene expression along with indicators of oxidative stress in placenta of TCE-exposed rats are consistent with the hypothesis proposed by Chia and co-workers that TET enzyme activation could be due to oxidative stress [57]. Rakoczy and co-workers previously reported modest but significant increases in *Tet* mRNA expression in mouse placenta [58]. However, unlike Rakoczy et al. who found increased *Tet1, Tet2, and Tet3,* we observed a significant increase in *Tet3* only. Because *Tet3* is involved in DNA demethylation in mouse zygotes [59, 60] and the placenta includes tissue of embryonic origin, it is interesting that TCE exposure induced placental *Tet3.* In contrast, *Tet2,* which is linked most strongly to cancer [61], was unchanged in placenta of our TCE-exposed rats. Although TCE exposure decreases DNA methylation in mouse liver [62], mouse cerebellum [63], and human hepatic L-02 cells [64], our study found no significant changes in DNA methylation (5-mC) in placentas of TCE-exposed rats. However, Jiang et al. found no TCE effect on global DNA methylation in mouse liver, similar to our result in rat placenta, but they did find DNA hypomethylation and hypermethylation of specific genes [65]. Future experiments could explore possible linkages between TCE exposure, ROS, 5-hmC, and gene expression, including validation of the 5-hmC results, to identify specific hydroxy-methylation changes of DNA in placental DNA. However, such experiments are beyond the scope of the current study.

Studies with autoimmune-prone MRL +/+ mice reported increased oxidative stress using TCE exposure at lower doses but longer exposure periods than in our study. Blossom et al. found decreased levels of GSH in cerebellum of MRL +/+ mice exposed to 0.1 mg/ml TCE in drinking water (~31 mg/kg/day) from gd 1 until postnatal day 42 [14]. A study by Wang et al. reported increased levels of oxidative stress lipid peroxidation markers including malondialdehyde (MDA) and 4-hydroxynonenal (HNE) in MRL +/+ mice exposed i.p. once

per week for 6 or 12 weeks with 10 mmol TCE /kg [66]. In another study, Wang et al. demonstrated significantly increased serum levels of MDA and HNE in female MRL +/+ mice exposed to 0.5 mg/ml TCE in drinking water for 48 weeks [67].

Oxidative stress in gestational tissues has been associated with several pathologies of human pregnancy, including preterm labor, preeclampsia, and fetal growth restriction [68, 69]. Furthermore, increased levels of the urinary oxidative stress markers 8-isoprostane and 8-OHdG in early or mid-gestation were associated with increased risk for preeclampsia and decreased gestational length, respectively, in a mostly Hispanic and African-American population of New Jersey [70]. Moreover, in a predominantly White Boston cohort with repeated measures of 8-isoprostane and 8-OHdG across gestation, average levels of urinary 8-isoprostane were associated with increased preterm birth, although increased urinary 8-OHdG was associated with decreased preterm birth [71]. Of particular interest, further statistical analysis of the latter Boston cohort found evidence for oxidative stress, as indicated by urinary 8-isoprostane, mediating associations between maternal phthalate exposure and preterm birth [72]. Because oxidative stress and inflammation are closely inter-related, we measured the pro-inflammatory cytokine IL-6 in maternal blood: there was considerable variability among the measures and differences between TCE-exposed and control rats were not statistically significantly different (Supplement Figure S3). There was lack of neutrophil infiltration in both the control and TCE-treated rat placental tissues (data not shown).

Although both rats and humans form hemochorial placentas in pregnancy, there are significant species differences. Whereas the yolk sac in women disappears by the end of the first trimester, rats develop an inverted yolk sac placenta early in pregnancy that provides critical nutrient transport until it ruptures and disappears closer to term around gd 17-18 [73]. With implantation in the rat occurring around gd 5, our TCE exposure period of gd 6-16 includes placentation from the early yolk sac placenta through establishment of the chorioallantoic placenta that begins around gd 11-12 [74]. Our observed effects of TCE on fetal growth and placental responses may have been due to directs effects on the fetus and placenta or indirect results of impacts on the yolk sac. Nonetheless, our exposure regimen provided sub-chronic TCE exposure over the period of critical placental development.

Inhalation predominates as the TCE exposure route in the workplace and can be an exposure route for the general population due to vapor intrusion [75]. TCE exposure via drinking water is an additional concern for the general public due to contamination from prior waste disposal practices [3]. TCE is among the most frequently detected contaminants of US public water supplies and US EPA National Priority List (commonly referred to as Superfund) sites [3], and is readily absorbed from the gastrointestinal tract of mice [76] and rats [77]. The US EPA Maximum Contaminant Level (MCL) for drinking water is 5 μg TCE/L [78], although exceedance of the MCL has been reported [3]. The TCE dose used in the present study is within an order of magnitude higher than would occur with occupational exposure at the US OSHA 100 ppm TCE permissible exposure limit (PEL) over an 8-h workday, but is several orders of magnitude higher than would be expected with environmental exposures from oral ingestion.

By administering TCE orally to rats on a wafer, our study has the advantage of using ingestion as a route of exposure relevant to humans. Although exposure via wafer may have introduced dose variability due to TCE evaporation, we minimized time for volatilization by presenting the wafer without TCE in a three-day training period, which allowed the rats to recognize the wafer as a treat and eat the wafer more quickly. Moreover, the low variability in maternal weights, fetal body weights, and maternal liver and kidney weights suggests that TCE evaporation from the wafer had minimal contribution to internal dose variation. Likewise, the low variability suggests that the statistically significant mean differences observed between TCE-exposed and control rats, though modest for some parameters, are meaningful indices of effect. Although measurement of TCE or its metabolites in serum and/or placenta was beyond the scope of the present investigation, collection of such data would have provided internal exposure verification and facilitated possible future comparisons with human studies. Furthermore, inclusion of fetal sex in future analyses of TCE effects on placenta is needed because of known sex-related differences in rat placental development [79]. It is possible that combining sexes contributed to the modest magnitude of some of the effects we observed. Finally, although our study provides a lowest observable effect level for TCE toxicity in the rat placenta and a potential underlying mechanism for adverse birth outcomes, future studies are needed to characterize the full dose-response relationship for TCE toxicity in placenta of pregnant rats including doses relevant to human environmental exposures.

5. Conclusions

In summary, TCE decreased fetal weight and increased biomarkers of oxidative stress that have been associated with adverse pregnancy outcomes in humans, including fetal growth restriction. Although oxidative stress is implicated as a mechanism involved in TCE-induced kidney and liver intoxication, to our knowledge, this is the first study that explores TCE-induced oxidative stress in the placenta in vivo. Results of the current study suggest that oxidative stress in the placenta is a possible mechanism relevant for epidemiological associations of TCE exposure and adverse birth outcomes [24, 39, 80]. Additionally, findings of increased levels of *Tet3* enzyme and 5-hmC suggest that exposure to TCE could modify DNA in placenta through an oxidative stress mechanism. However, because it is likely that oxidative stress is not unique to the placenta given prior study observations in non-gestational tissues, other factors may contribute to fetal growth deficits and further studies are needed to validate this and other possible mechanisms by which TCE exposure may lead to fetal growth restriction in humans. Nonetheless, our findings contribute to the weight of evidence for TCE-induced adverse pregnancy outcomes, providing new insight into TCE effects on the placenta.

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Abbreviations

BCA	bicinchoninic acid
CYP	cytochrome
DCVC	S-(1,2-dichlorovinyl)-L-cysteine
ELISA	enzyme-linked immunosorbant assay
gd	gestational day
GGT	γ-glutamyltransferase
GSH	glutathione
GST	glutathione S-transferase
5-hmC	5-hydroxymethylcytosine
8-OHdG	8-hydroxy-deoxyguanosine
IL	interleukin
5-mC	5-methylcytosine
PBS	phosphate-buffered saline
qRT-PCR	real time quantitative reverse transcription PCR
TCE	trichloroethylene
TET	ten-eleven translocation

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Highlights

- Trichloroethylene exposure during pregnancy decreased fetal weight in rats
- Trichloroethylene exposure increased markers of oxidative stress in rat placenta
- Trichloroethylene exposure increased rat placental mRNA expression of *Tet3*

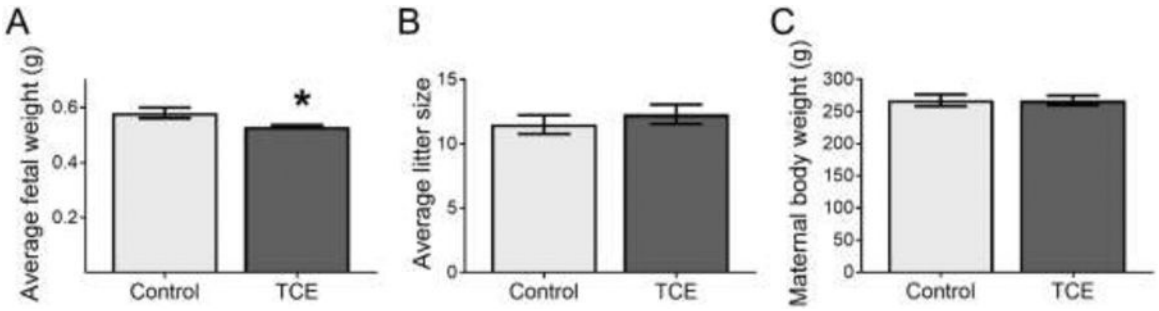

Fig. 1.

Indicators of fetal and maternal health on gd 16 of rats exposed to 0 (control) or 480 mg TCE/kg-day from gestational day 6-16. N=10 rats per group. The bars indicate means ± SEM. **A)** Average fetal weight per litter. Fetal weights were averaged per litter and a grand mean was calculated for statistical comparison. **B)** Litter size (number of fetuses per litter). **C)** Maternal body weight. *Statistically significantly different ($p<0.05$).

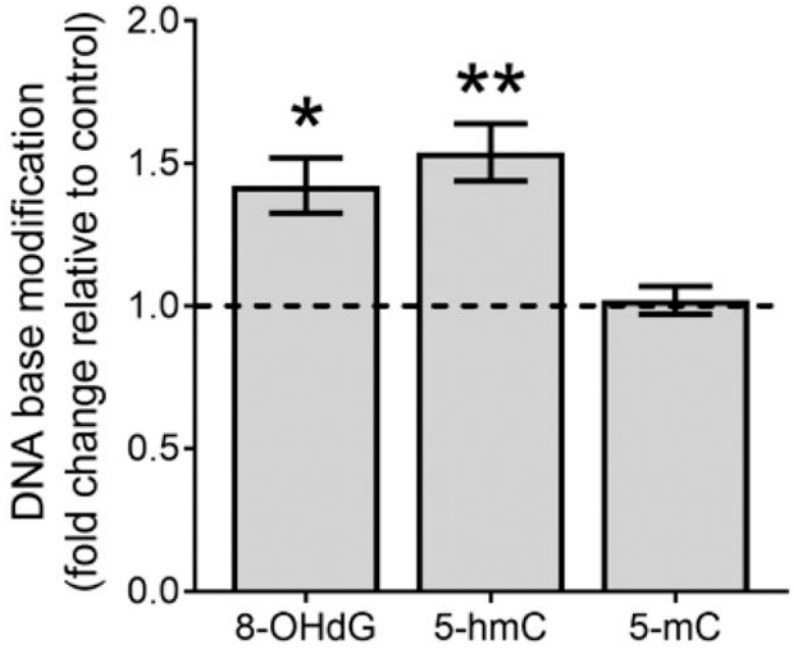

Fig. 2.

Placental DNA modification by TCE: changes in 8-hydroxy-deoxyguanosine (8-OHdG), 5-hydroxymethylcytosine (5-hmC), and 5-methylcytosine (5-mC). Placentas were analyzed on gd 16 of control rats (N=7) and rats exposed to 480 mg TCE/kg-d (N=8) from gd 6-16. Samples from 3 placentas per litter were assayed. The data were statistically analyzed on a per litter basis and are shown as mean ± SEM. * The horizontal dashed line indicates control value = 1. Significantly increased compared with control (p=0.02). **Significantly increased compared with control (p=0.005).

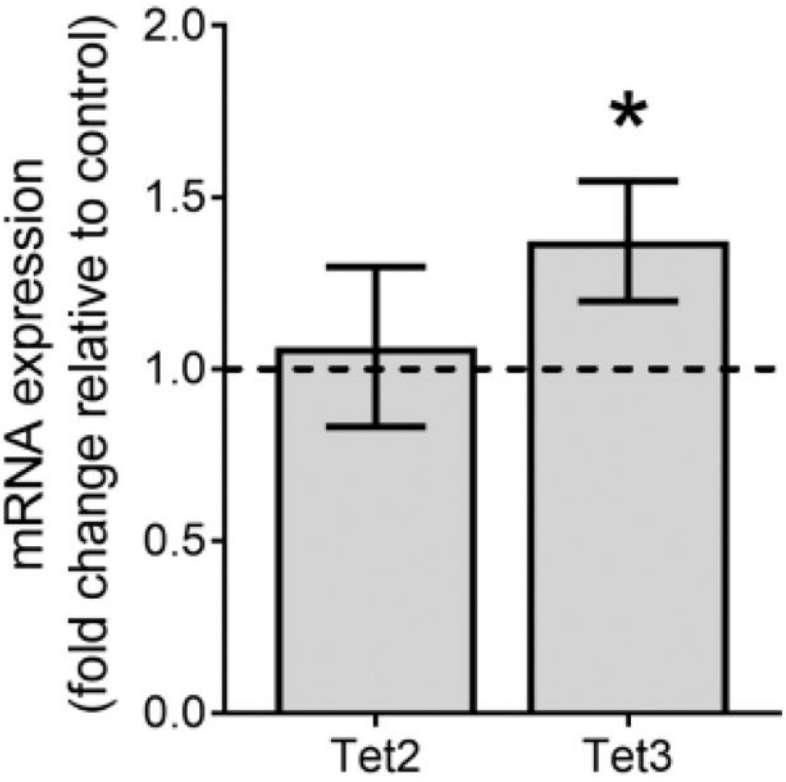

Fig. 3.

TCE-induced change of ten eleven translocation (*Tet*) mRNA expression levels in placenta. Placentas were analyzed on gd 16 of control rats (N=7) and rats exposed to 480 mg TCE/kg-d (N=8) from gd 6-16. Samples from 3 placentas were pooled within litter for analysis. The horizontal dashed line indicates control value = 1. *Significantly increased compared with control ($p<0.05$).

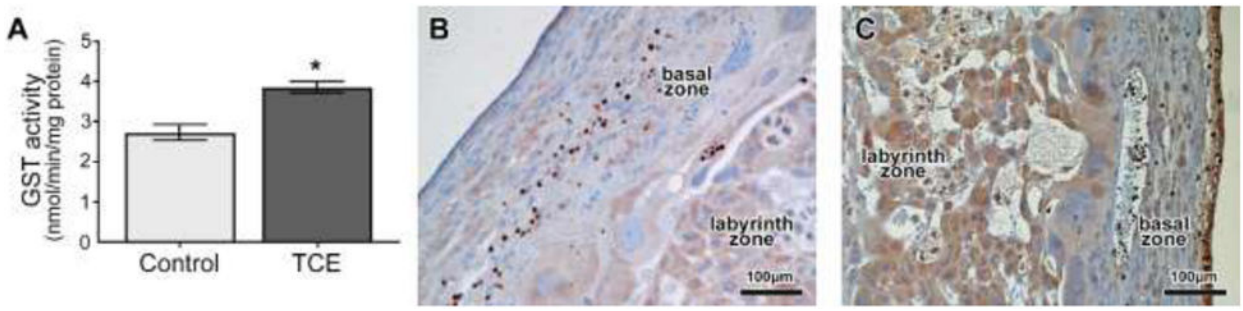

Fig. 4.

TCE effects on glutathione S-transferase (GST) in gd 16 placentas of control rats or rats exposed from gd 6-16 to 480 mg/kg TCE/kg-day. **A)** Activity of placental GST. Data are shown as means ± SEM. N=4 rats per group, with 3 placentas/litter. *Significantly increased compared with vehicle control (p=0.02). **B and C)** Immunohistochemical staining for GST-pi in representative images of left labyrinth and basal zones on the fetal side of placenta from control (**B**) and TCE-exposed (**C**) rats, showing increased intensity of staining throughout the labyrinth and basal zones of the placenta in the TCE-exposed placenta.